Virology 289, 343–352 (2001)
doi:10.1006/viro.2001.1123, available online at http://www.idealibrary.com on IDEAL®

Mutational Analysis of the Membrane Proximal Heptad Repeat of the Newcastle Disease Virus Fusion Protein

Lori W. McGinnes, Theresa Sergel, Hong Chen, Ludwig Hamo, Steve Schwertz, Donghui Li, and Trudy G. Morrison[1]

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01532

Received April 10, 2001; returned to author for revision July 10, 2001; accepted July 28, 2001

Paramyxovirus fusion proteins have two heptad repeat domains, HR1 and HR2, that have been implicated in the fusion activity of the protein. Peptides from these two domains form a six-stranded, coiled-coil with the HR1 sequences forming a central trimer and three molecules of the HR2 helix located within the grooves in the central trimer (Baker *et al.,* 1999, *Mol. Cell* 3, 309; Zhao *et al.* 2000, *Proc. Natl. Acad. Sci. USA* 97, 14172). Nonconservative mutations were made in the HR2 domain of the Newcastle disease virus fusion protein in residues that are likely to form contacts with the HR1 core trimer. These residues form the hydrophobic face of the helix and adjacent residues ("a" and "g" positions in the HR2 helical wheel structure). Mutant proteins were characterized for effects on synthesis, steady-state levels, proteolytic cleavage, and surface expression as well as fusion activity as measured by syncytia formation, content mixing, and lipid mixing. While all mutant proteins were transport competent and proteolytically cleaved, these mutations did variously affect fusion activity of the protein. Nonconservative mutations in the "g" position had no effect on fusion. In contrast, single changes in the middle "a" position of HR2 inhibited lipid mixing, content mixing, and syncytia formation. A single mutation in the more carboxyl-terminal "a" position had minimal effects on lipid mixing but did inhibit content mixing and syncytia formation. These results are consistent with the idea that the HR2 domain is involved in posttranslational interactions with HR1 that mediate the close approach of membranes. These results also suggest that the HR2 domain, particularly the carboxyl-terminal region, plays an additional role in fusion, a role related to content mixing and syncytia formation.

INTRODUCTION

Entry of enveloped viruses requires the fusion of viral membranes with cellular membranes, a process directed by membrane-associated viral fusion proteins. Viral fusion proteins have sequence elements important in fusion including fusion peptide and heptad repeat (HR) domains. Fusion peptides are domains proposed to insert into target or cellular membranes disordering the lipids in those membranes and attaching the protein to target membranes (Hernandez *et al.,* 1996). HR domains have been implicated in pulling target and attack or virion membranes into close proximity required for subsequent membrane fusion (Weissenhorn *et al.,* 1999).

The *Paramyxoviridae* fusion (F) proteins are synthesized as precursors, F_0, and proteolytic cleavage to the disulfide linked F_1 and F_2 polypeptides is required for fusion activity of the protein. This cleavage places the F protein fusion peptide at the amino terminus of F_1. Adjacent to the fusion peptide is a heptad repeat region (HR1), which mutational analysis has shown to be important in the fusion activity of the protein (Sergel-Germano *et al.,* 1994). Paramyxovirus F proteins also have a heptad repeat domain (HR2) adjacent to the transmembrane domain of the F_1 protein. The HR2 domain has also been implicated in the fusion activity by mutational analysis as well as analysis of peptides with sequences of the HR2 domain (Buckland *et al.,* 1992; Ghosh *et al.,* 1998; Lambert *et al.,* 1996; Rapaport *et al.,* 1995; Reitter *et al.,* 1995; Wild and Buckland, 1997; Yao and Compans, 1996; Young *et al.,* 1997). HR2 peptides inhibit fusion and also form complexes with peptides from the HR1 domain (Joshi *et al.,* 1998; Young *et al.,* 1999) to form a six-stranded structure with a central core trimer of HR1 peptides with three HR2 peptides bound to the trimer surface (Baker *et al.,* 1999; Zhao *et al.,* 2000). These results have been interpreted to indicate that HR2 peptides mimic the HR2 domain in the intact protein, interfering with interactions of the HR2 domain with HR1 resulting in fusion inhibition. One interpretation of this inhibition is that the HR1 domain is not complexed with the HR2 domain prior to activation of fusion and is accessible to HR2 peptide binding. The recently published crystal structure of the Newcastle disease virus (NDV) F protein does not clarify this issue since neither the HR2 domain nor the amino-terminal part of the HR1 domain was visible in the structure (Chen *et al.,* 2001).

These considerations suggest that the F protein is synthesized in a prefusion conformation that changes upon activation of fusion. Fusion activation requires

[1] To whom correspondence and reprint requests should be addressed. Fax: (508) 856-5920. E-mail: trudy.morrison@umassmed.edu.

0042-6822/01 $35.00

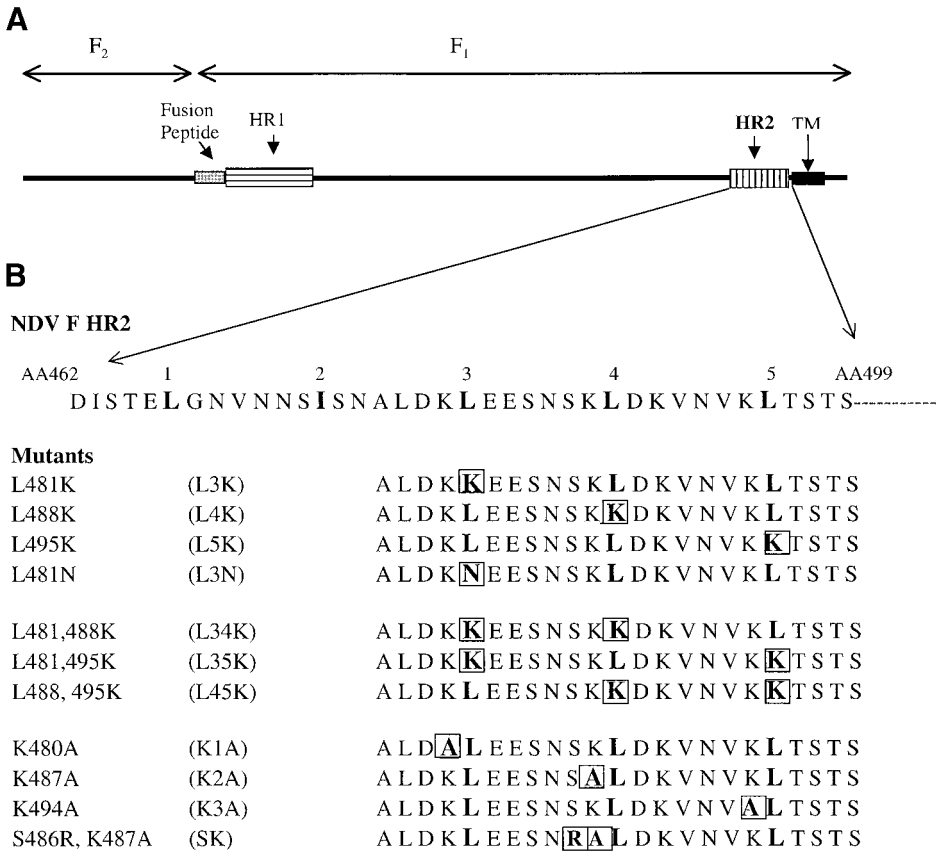

FIG. 1. Description of mutants. (A) A diagram of the F protein sequence indicates the approximate location of important sequence elements related to the fusion activity of the protein. (B) The sequence of the HR2 domain of the NDV F protein, from amino acid 462 to 499, is shown. The five "a" position residues in the NDV HR2 heptad repeat are indicated by numbers above the sequence. Also shown are the sequences of the HR2 domain of the mutants characterized. The mutants are identified by the position of the amino acid in the F sequence as well as an abbreviation in parentheses.

cleavage of the molecule that is accompanied by a conformational change (Hsu *et al.,* 1981). Additional conformational changes with onset of fusion are likely, changes that are analogous to those detected upon acid activation of the influenza HA protein (Carr and Kim, 1993; Hernandez *et al.,* 1996) or to changes detected in the retroviral envelope proteins upon attachment to receptors (Chan and Kim, 1998; Furuta *et al.,* 1998; Hernandez *et al.,* 1997; Tan *et al.,* 1997; Wiessenhorn *et al.,* 1997). To understand structures of the prefusion and postfusion conformations of the NDV F protein, we have extended our previous mutational analysis of the HR2 domain (Reitter *et al.,* 1995) with a focus on nonconservative changes of residues that may be important to HR1 domain interactions. All mutant proteins resulted in transport competent proteins, suggesting that normal complex formation between HR1 and HR2 domains is not necessary for folding of the prefusion form of the protein. These mutations did, however, variously affect fusion activity of the protein. We have found that single, nonconservative mutations can significantly inhibit fusion. Furthermore, the position of the change within the heptad repeat is important for the degree of inhibition and the step in the fusion process that is inhibited. Results suggest that the HR2 domain may be involved in steps subsequent to the close approach of two membranes.

RESULTS

Mutagenesis of the HR2 domain

Figure 1A shows a diagram of the linear sequence of the NDV F protein with the HR domains indicated. The HR2 domain is minimally defined here as the region containing the heptad repeat of leucine/isoleucine residues. Figure 1B shows the sequence of the NDV F protein in this minimal HR2 domain with leucine/isoleucine residues forming the heptad repeat shown in boldface. We have focused on the carboxyl-terminal region of the HR2 domain (from the third to fifth leucine residue), introducing nonconservative mutations in residues that potentially form contacts with the HR1 domain. The sequences of mutants characterized below are shown in Fig. 1B. Leucine residues, in the "a" position of the heptad repeat, were changed to lysine singly or doubly. In addition, the third leucine (position 481) was also changed to asparagine (L3N). Lysine residues in the "g" positions were changed individually to alanine to generate K1A, K2A, and K3A mutant proteins. A double mutant (SK)

substituted the serine at 486 with arginine and the lysine at 487 with alanine.

Expression of mutant proteins

The mutant proteins were expressed in Cos-7 cells using a simian virus 40-based vector and radioactively labeled with [^{35}S]methionine for 2 h at 48 h posttransfection as previously described (Sergel *et al.,* 1993a,b; Sergel-Germano *et al.,* 1994). The labeled proteins, precipitated with anti-Ftail polyclonal antibody (Wang *et al.,* 1992), were electrophoresed in the presence (Fig. 2A) of reducing agent. Under these conditions, the F_0 and F_1 proteins were resolved while F_2 protein is not detected (Sergel-Germano *et al.,* 1994). All the L to K ("a" position residues) mutant proteins were expressed at levels comparable to wild-type F protein and all mutant proteins were proteolytically cleaved as efficiently as the wild-type protein. Similar results were obtained with the K to A ("g" position residues) mutant proteins (not shown). Interestingly, all proteins that contained an alteration in the leucine at amino acid 481 (L3) migrated slightly slower than wild-type protein as did protein with a mutation at the adjacent lysine residue at amino acid 480 (K1A) (Fig. 2C). This phenomenon was also noted for proteins with a leucine to alanine change at the same position (Reitter *et al.,* 1995). The reason for this difference from wild-type protein is unknown.

The steady-state levels of mutant proteins were assessed by Western analysis (Figs. 2B and 2C). As previously noted, detection of NDV F protein by Western analysis is successful only if proteins are not boiled prior to electrophoresis (Sergel-Germano *et al.,* 1994). All mutant proteins, including those with mutations in the "a" position (Fig. 2B) as well as those with mutations in the "g" position (Fig. 2C), were detected at levels comparable to wild type. Thus none of the mutations significantly increased the susceptibility of the protein to proteolytic degradation. However, proteins with mutations at leucine 481 (L3) migrated as an oligomeric polypeptide with little detectable material in the monomer region of the gel. Sodium dodecyl sulfate (SDS)-resistant oligomeric structures formed by paramyxovirus F proteins have been reported previously (Collins and Mottet, 1991; Sergel-Germano *et al.,* 1994) and can be seen in the lane containing wild-type protein (Fig. 2B). However, the mutation in L3 resulted in a protein that was exclusively in this complex, a finding that suggests that these mutant proteins are in a conformation somewhat different than wild-type protein.

Surface expression

Proteolytic cleavage of all mutant proteins suggested that these proteins were transported to the *trans*-Golgi membranes (Doms *et al.,* 1993) and, therefore, likely to the cell surface. This conclusion was confirmed by flow

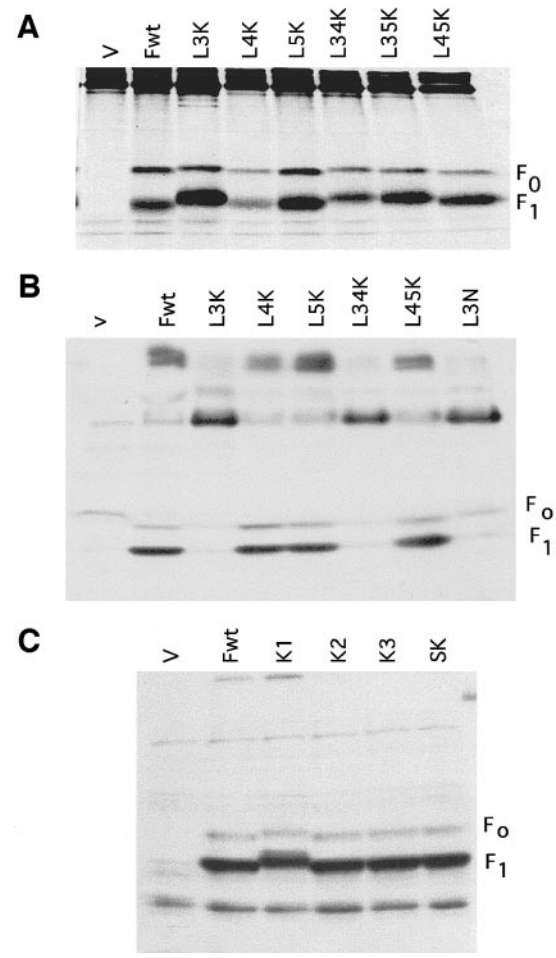

FIG. 2. Expression of mutant proteins. (A) Precipitation of radiolabeled F proteins synthesized in cells transfected with plasmids encoding the mutant F proteins indicated above each lane. Cells transfected with 0.5 μg of DNA/35-mm plate of Cos-7 cells were labeled with [^{35}S]methionine for 2 h at 48 h posttransfection and subjected to a 4-h chase. Proteins present in extract from 2×10^5 cells were precipitated with anti-Ftail antibody and the precipitated proteins were resolved on polyacrylamide gels in the presence of reducing agent. Results with L3N protein (not shown) were identical to the results with L3K protein. (B and C) Results of Western analysis of mutant proteins (indicated above each lane) under reducing conditions present in 5×10^5 cells at 48 h posttransfection. Results with L35K protein (not shown) were identical to results with L34K protein. Anti-Ftail was used to detect F protein. Positions of F_0, uncleaved F protein, and F_1, cleaved F protein, were determined by marker infected cell extracts electrophoresed in parallel; V, vector.

cytometry. Cells were transfected with suboptimal levels of DNA in order to avoid effects due to overexpression of viral proteins, and the amounts of DNA used were carefully controlled in order to compare accurately expression levels. Comparisons of mutants with changes at "a" positions with wild type showed that the number of positive cells detected for each mutant transfection was 1.2 to 1.5 times greater than wild type (Fig. 3). The numbers of positive cells expressing mutants with K to A changes and the SK mutant were virtually identical to wild type. Interestingly, Fig. 3 also shows that the inten-

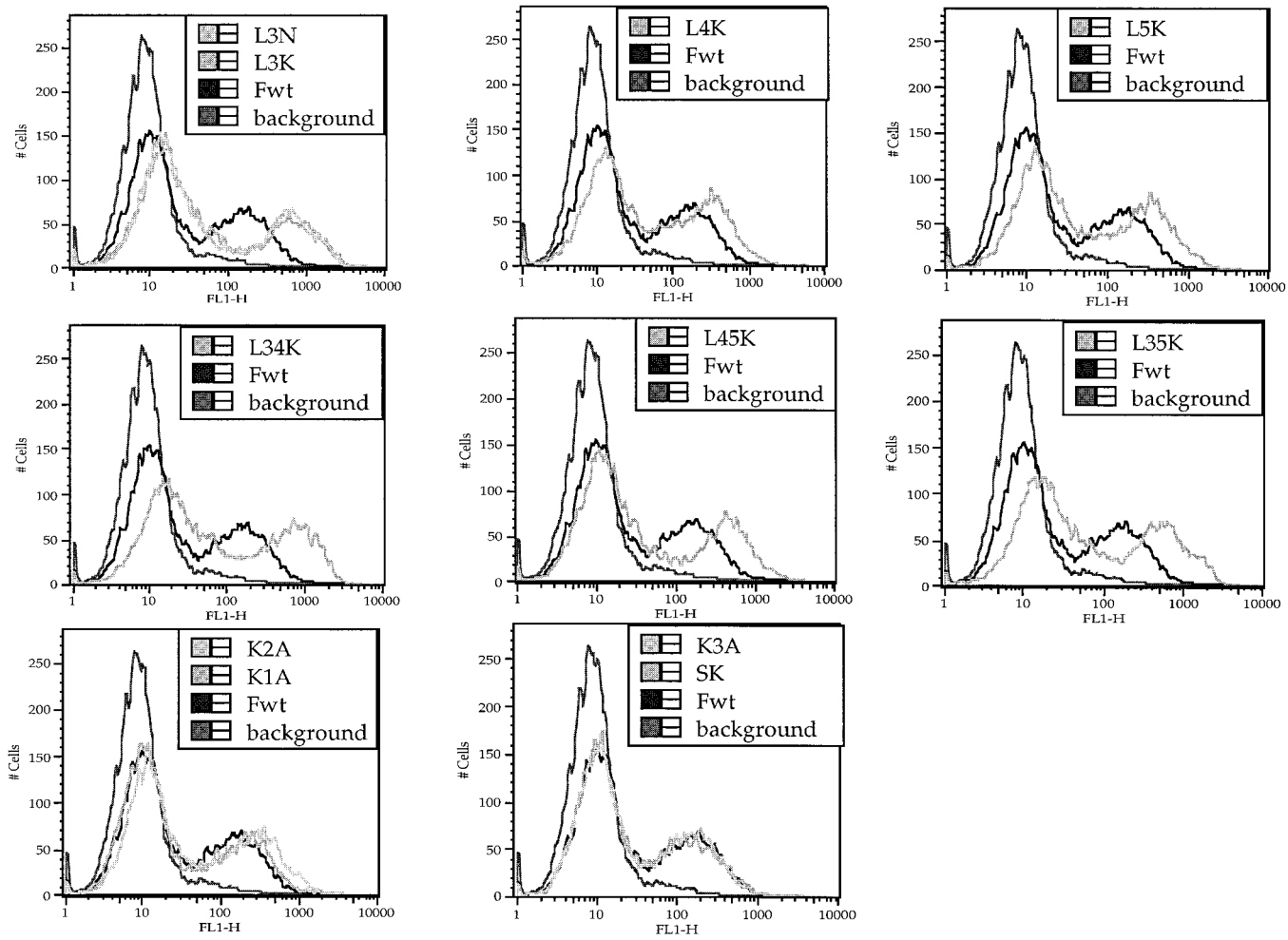

FIG. 3. Analysis of surface expression of mutant proteins by flow cytometry. Cells transfected with 0.5 μg DNA/35-mm plate were processed for analysis by flow cytometry as described under Materials and Methods. The primary antibody was anti-NDV antibody. Each panel shows background (cells transfected with vector alone and incubated with both primary and secondary antibodies) and cells expressing wild-type F protein as well as cells expressing a mutant F protein. The figure shows the results of one experiment. Identical results were obtained in a duplicate experiment using different preparations of mutant DNA. FL1-H, fluorescence gated for Alexa-488-labeled antibody.

sities of fluorescence signal on cells transfected with the L to K mutants were significantly greater than that detected for wild type. The largest increases were detected with cells expressing mutations in the L3 position or in cells expressing the double mutant, L45K. Increases in intensity of signal were not detected for K to A mutants or the double mutant SK with the exception of K2A, which yielded a slight increase in intensity of signal (Fig. 3). The reasons for increased intensity of signal are unknown.

Fusion activity of mutant proteins

To measure the ability of these mutant proteins to direct membrane fusion, Cos-7 cells were transfected with only plasmids encoding mutant fusion proteins or with plasmids encoding mutant fusion proteins as well as a plasmid encoding the wild-type HN protein. The fusion activities of these mutant proteins were quantitated by syncytia size and frequency (Table 1), as previously described (Sergel-Germano *et al.,* 1994). None of the F mutants were capable of significant syncytia formation in the absence of HN protein expression (not shown). In the presence of HN protein expression, the K to A mutants and the SK mutant directed nearly wild-type levels of syncytia formation. However, the syncytia sizes directed by the L3K, L3N, and L5K mutant proteins were very small and, importantly, the frequencies of these small syncytia were quite low (Table 1). The syncytia size and frequency directed by the L4K mutant protein activity were reduced to some extent. The syncytia-forming activities of the double mutant L35K and L45K proteins were also minimal, while the L34K mutant protein directed the formation of small syncytia but at reduced efficiency. The properties of the L34K mutant were surprising since a protein in which the L3 and L4 positions are both substituted with alanine did not direct syncytia formation (Reitter *et al.,* 1995).

TABLE 1

Fusion Activity of Mutant Proteins

DNA	Syncytia size (percent of wild type)	Syncytia frequency (percent of wild type)	Content mixing (percent of wild type)
A.			
HN+Fwt	100	100	100
Fwt	5 (±9)	3	1.8 (±1)
HN	5 (±4)	2	5 (±2)
B.			
HN DNA +:			
Fwt	100	100	100
L3K	30 (±1.4)	20	12.5 (±8)
L3N	33 (±20)	20	7 (±0.5)
L4K	47 (±7)	115	90 (±40)
L5K	20 (±5)	12	33 (±2)
L34K	43 (±15)	40	14 (±1)
L35K	18 (±11)	6	3.8 (±3)
L45K	18 (±13)	10	6.5 (±2)
K1A	77 (±5)	100	125 (±5)
K2A	88 (±10)	100	235 (±3)
K3A	73 (±10)	100	127 (±4)
SK	100 (±10)	100	115 (±4)

Note. A. Syncytia size, syncytia frequency, and content mixing activities directed by F wild-type protein or HN protein expressed alone are shown as a percentage of values obtained with wild-type F protein expressed with HN protein. B. Syncytia size, syncytia frequency, and content mixing activities directed by each mutant F protein cotransfected with wild-type HN protein are expressed as a percentage of values obtained with wild-type F protein coexpressed with HN protein. Syncytia size and frequency were determined as described under Materials and Methods. Content mixing was measured as β-galactosidase activity as described under Materials and Methods.

Fusion activities of mutant proteins were also assayed by measuring content mixing of fusing cells as described under Materials and Methods. Content mixing was measured by β-galactosidase activity that could be expressed only upon cell fusion. Cells transfected with a plasmid encoding the β-galactosidase gene under the control of a tet-sensitive transactivator were mixed with cells transfected with HN and F cDNAs as well as a plasmid encoding the tet-responsive transactivator of transcription. β-Galactosidase synthesis was induced only when cells from the different populations fused. This assay is specific for fusion directed by the HN and F proteins as little enzyme activity was present when only F cDNA was present or when only HN cDNA was present (Table 1). Furthermore, there was little activity after transfection with HN and an F cDNA that encoded an uncleaved F protein (McGinnes *et al.,* 2001). This assay is also dependent upon the levels of expression of F proteins (McGinnes *et al.,* 2001).

Content mixing activities of the double mutants were significantly reduced from wild type. Similarly, single mutations at the L3 site or the L5 site also inhibited content mixing while the L4K mutation had little effect on this activity (Table 1). However, the content mixing activities of the K to A mutant proteins were very similar to that of the wild-type protein as would be expected from syncytia-forming activities of these proteins.

To determine whether mutants that had little or no ability to direct content mixing were capable of mediating lipid mixing or hemifusion, red blood cells (RBC), labeled with the fluorescent probe R18, were incubated with cells expressing these mutant proteins as previously described (Kemble *et al.,* 1993, 1994). Figure 4 shows representative results. As expected, cells expressing the HN protein bound red blood cells but there was no transfer of fluorescence from the RBC to the cells. In contrast, cells expressing both HN and F proteins became fluorescent as a result of the dye transfer. Cells expressing L3K and L3N were defective in hemifusion, as was the double mutant L35K. The L5K mutant protein as well as the L45K mutant protein showed significant hemifusion activity.

DISCUSSION

Several laboratories have reported properties of paramyxovirus F proteins with conservative mutations of hydrophobic residues in the "a" positions of the HR2 domain. It was shown that at least two of these residues had to be changed to significantly affect fusion activity (Buckland *et al.,* 1992; Reitter *et al.,* 1995) although the stage of fusion affected by these mutations was not reported. No change inhibited proteolytic cleavage or surface expression (Reitter *et al.,* 1995). To extend these studies, we characterized the properties of nonconservative changes in residues on the hydrophobic face of the helix as well as residues in the adjacent "g" positions.

Effects of mutations on intracellular processing and surface expression of the F protein

The role of the HR2 domain in the formation of the prefusion paramyxovirus F protein is unknown even in the light of the recently published structure of the NDV F protein (Chen *et al.,* 2001). In this structure, the HR2 domain was missing and, therefore, its disposition in the structure was unclear. Our analysis of mutants with single or double nonconservative changes in leucine residues in the "a" position of the HR2 helix showed that no change inhibited proteolytic cleavage or surface expression. In addition to the heptad repeat of leucine/isoleucine residues, the NDV F protein in all strains of virus has a striking heptad repeat of lysine residues that fall in the "g" position of the helical structure of the HR2 domain. Mutation of these residues also did not inhibit proteolytic cleavage and levels of surface expression. We have previously described properties of mutants with nonconservative changes that fall on the more charged side of the helix (in the "b" and "c" positions of the helix) and

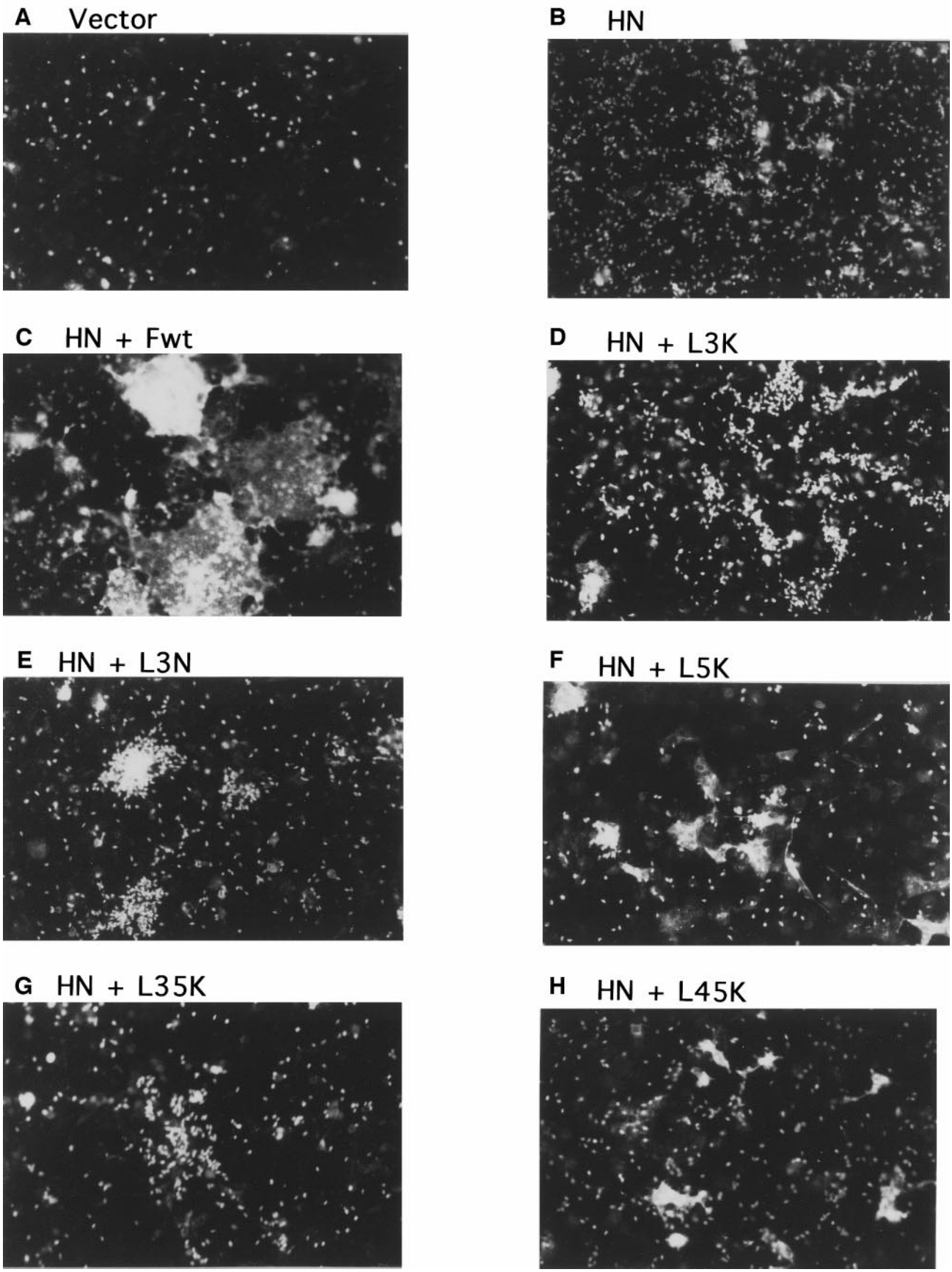

FIG. 4. Lipid mixing directed by mutant proteins. Avian red blood cells, labeled with R18 as described under Materials and Methods, were added to cells transfected with wild-type HN protein cDNA and wild-type or mutant F protein cDNA. After incubation at 37°C for 1 h, monolayers of cells were washed to remove unbound RBC and photographed through a Nikon fluorescence microscope.

found that none of these significantly inhibit cleavage or surface expression (Reitter *et al.,* 1995). The NDV F protein HR2 domain also has a used glycosylation addition signal in position "e" of the helix (McGinnes *et al.,* 2001). Elimination of this carbohydrate by mutation of the addition signal had only a small effect on the surface expression of the protein (McGinnes *et al.,* 2001). Thus no mutation in the NDV F protein HR2 domain, conservative or nonconservative, significantly inhibited surface expression, suggesting that the conformation of this domain may not be important for the formation of a transport competent, prefusion form of the protein.

Effects of mutations on fusion activity of the F protein

Fusion mediated by viral glycoproteins is thought to proceed through several steps initiated by the close approach of attack and target membranes followed by hemifusion or the fusion of the outer leaflets of the two membranes, pore formation, and pore expansion and syncytia formation (Hernandez *et al.,* 1996). The complexes formed between peptides from the HR1 and HR2 domains have been interpreted to mimic a structure in the intact protein that mediates the close approach of the target and attack membranes. It has been proposed that formation of this complex, upon activation of the F protein, serves to initiate fusion (Baker *et al.,* 1999; Matthews *et al.,* 1994; Skehel and Wiley, 1999, 2000; Weissenhorn *et al.,* 1999). Thus inhibition of this complex should inhibit fusion at the earliest stage, that of close approach.

The structures of the complexes formed by peptides from the HR1 and HR2 domains of the simian virus 5 and respiratory syncytial virus F proteins suggest that the "a" position residues contact the HR1 core trimer (Baker *et al.,* 1999; Zhao *et al.,* 2000). Residues in the "e" and "g" position also potentially influence the interaction. Thus double and perhaps single nonconservative changes in these residues should interfere with the interaction of the HR1 and HR2 domains and, therefore, block hemifusion as well as content mixing and syncytia formation.

Mutations in the HR2 domain potentially also affect interactions with the HN protein. As with most paramyxovirus F proteins, fusion activity requires the coexpression of HN protein (Lamb, 1993). Abundant evidence suggests an interaction between the two proteins that is required for fusion (Hu *et al.,* 1992). While indirect evidence implicates minimally the stalk domain of the HN protein in these interactions (Deng *et al.,* 1995; Tanabayashi and Compans, 1996; Tsurudome *et al.,* 1995), the domains of F protein involved in these interactions are not clear. If, however, the HR2 domain interacts with the HN protein, then HR2 domain mutations may also affect fusion by interfering with interactions with the HN protein. Because the role of HN protein in the fusion activity of F protein is unclear, it is difficult to predict the step of fusion that might be inhibited if there is a defect in HN–F protein interactions.

Nonconservative mutants with changes in the "g" position of the helix had no apparent effect on the fusion activity of the protein, suggesting that these residues are minimally involved in HR1 interactions in the NDV F protein or with HN protein. However, changes in "a" position residues did impact fusion activity of the protein. In contrast to previously reported results obtained with conservative mutations (Buckland *et al.,* 1992; Reitter *et al.,* 1995), single nonconservative changes in "a" position residues interfered with fusion activity. However, the position of the residue is important to the degree and type of effect. Alteration of the third leucine, which is in the middle of the HR2 domain, had the most effect on fusion. Hemifusion was significantly inhibited as were subsequent stages in fusion, a result consistent with inhibition of the close approach of membranes due to a defective HR1–HR2 interaction. Alteration of the L4 residue had minimal effect on the activity of the protein, suggesting that this residue has only a small effect on HR1–HR2 complex formation or HN protein interactions. Alteration of the L5 residue or the L4 and L5 residues together also inhibited syncytia formation and content mixing but minimally affected hemifusion. This phenotype might be accounted for by inefficient or less stable HR1–HR2 complex formation. Alternatively, the L4 and L5 residues may be less important in HR1–HR2 complex formation, but may be more important in pore formation. That HR2 mutations, L5K and L45K, allow hemifusion (therefore, close approach) but are blocked at subsequent steps suggests that the HR2 domain is also important in these subsequent steps. These results suggest that the HR2 domain is not only involved in the close approach of two membranes but also plays an additional role in fusion, a role in steps related to content mixing and syncytia formation.

MATERIALS AND METHODS

Cells and vectors

Cos-7 cells, obtained from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL/Life Technologies) supplemented with nonessential amino acids, vitamins, penicillin/streptomycin, and 10% fetal calf serum. NDV (strain AV) HN and F protein genes were expressed in Cos-7 cells using pSVL (Amersham Pharmacia Biotech) as previously described (Sergel-Germano *et al.,* 1994).

Site-specific mutagenesis

The F gene mutants were generated with the Sculptor mutagenesis kit from Amersham Pharmacia Biotech Corp. using protocols specified by the manufacturer. Mutant oligonucleotides had appropriate codon changes

and were 25 to 30 bases in length. The entire gene of each mutant F protein DNA was sequenced to verify that no additional mutations were introduced into the sequence by the mutagenesis protocols. The mutations isolated are shown in Fig. 1. Mutants are named, in single-letter code, with the amino acid in wild type, the position of the change, and the amino acid in the mutant.

Transfections

Transfections using lipofectamine (Gibco BRL/Life Technologies) were done essentially as recommended by the manufacturer. Cos-7 cells were plated at 3×10^5 per 35-mm plate. Twenty to 24 h later, the cells were transfected. For each 35-mm plate, a mix of DNA (0.1–1 μg depending upon the experiment) in 0.1 ml OptiMem (Gibco BRL/Life Technologies) and 10 μl of lipofectamine in 0.2 ml of OptiMem was incubated at room temperature for 40 min, diluted with 0.7 ml OptiMem, and added to a plate previously washed twice with 2 ml of OptiMem. Cells were incubated for 5 h, DNA–lipofectamine complexes were removed, and then 2 ml of supplemented DMEM was added.

Antibodies

Antibodies used were anti-Ftail and anti-NDV. Anti-Ftail antibody was raised against a synthetic peptide with the sequence of the cytoplasmic tail of the fusion protein as described by Wang *et al.* (1992) and prepared by the Peptide Core Facility of the University of Massachusetts Medical School. Anti-NDV is a polyclonal antiserum raised in rabbits against UV inactivated virions as previously described (Sergel *et al.,* 1993b).

Radiolabeling and immunoprecipitation of protein

Transfected cells were radiolabeled for 2 to 4 h at 37°C in DMEM lacking methionine but containing 100 μCi of [^{35}S]methionine (Amersham Pharmacia Biotech) per milliliter. At the end of the labeling period, cells were washed in phosphate-buffered saline (PBS) and lysed in RSB buffer (0.01 M Tris–HCl, pH 7.4, 0.01 M NaCl) containing 1% Triton X-100 and 0.5% sodium deoxycholate, 2 mg/ml iodoacetamide, and 0.2 mg/ml DNase as previously described (Sergel *et al.,* 2000). Immunoprecipitation of NDV proteins was accomplished as previously described (Sergel *et al.,* 1993b).

Western analysis of mutant proteins

Freshly prepared, unfrozen cell extracts prepared as described for immunoprecipitation were diluted in sample buffer and loaded onto 10% polyacrylamide gels without boiling. After electrophoresis, the gels were subsequently equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 5% methanol, pH 8.2) and transferred to Immobilon-P (Millipore Corp.) membranes. The membrane was blocked in PBS containing 0.5% Tween 20 and 10% nonfat dried milk for 2 h at room temperature or overnight at 4°C. Membranes were washed in PBS containing 0.5% Tween 20 and incubated with primary antibody diluted in PBS containing 0.5% Tween 20 and 0.5% nonfat milk for 2 h at room temperature. Membranes were washed and then incubated in secondary antibody, anti-rabbit IgG coupled to horseradish peroxidase (Boehringer Mannheim) diluted in PBS containing 0.5% Tween 20 and 0.5% nonfat milk, for 1 h at room temperature. Membranes were washed extensively and bound antibody was detected using the ECL Western blotting detection reagent system (Amersham Pharmacia Biotech).

Flow cytometry

Cells transfected with 0.5 μg plasmid DNA/35-mm plate were removed from plates with Cell Dissociation Buffer (Sigma Co.) after a 1-min pulse in trypsin (50 μg/ml), washed in PBS containing 1% bovine serum albumin and 0.02% azide (FACS buffer), and incubated with anti-NDV antibody in FACS buffer for 1 h at 4°C. After cells were washed three times with FACS buffer, cells were incubated for 1 h at 4°C in FACS buffer with goat anti-rabbit IgG coupled to Alexa dye 488 (Molecular Probes). After three washes in FACS buffer, cells were resuspended in PBS containing 2% paraformaldehyde and subjected to flow cytometry (University of Massachusetts Medical School Flow Cytometry Facility). All experiments included cells transfected with vector alone and incubated with both primary and secondary antibodies.

Fusion assays

Syncytia formation. Cos-7 cells were cotransfected with wild-type or mutant fusion protein genes and the wild-type HN protein gene. At 48 h posttransfection, the number of nuclei in 40 syncytia were counted to determine the average size of syncytia at each time point as previously described (Sergel *et al.,* 1993b). Values obtained after transfection of the vector alone were subtracted. The frequency of syncytia formation was determined by averaging the number of syncytia containing five or more nuclei in 20 fields.

Content mixing. To measure content mixing, a plasmid encoding a tet-responsive transcriptional activator, pTet-Off (Clontech), was transfected alone with HN and F cDNAs. A separate population of cells was transfected with pTRE2 (Clontech) with a β-galactosidase gene inserted into the cloning cassette. This plasmid contains a tet-responsive element upstream from a CMV promoter. After 20 h cells were trypsinized and Cos-7 cells transfected with the β-galactosidase gene were mixed with cells transfected with HN and F DNAs as well as the plasmid pTet-Off and plated at subconfluent concentra-

tions. Syncytia began to form at 40–42 h posttransfection as cells became confluent. At 50 h posttransfection, when extensive syncytia formation was evident, the monolayers were lysed (Reporter Lysis Buffer, Promega), and extracts were assayed for β-galactosidase activity. Cell extract diluted with Reporter Lysis Buffer was incubated with equivalent volumes of 2× assay buffer (0.2 M sodium phosphate, pH 7.3, 1.3 mg/ml *o*-nitrophenyl β-D-galactopyranoside, 2 mM $MgCl_2$, 100 mM β mercaptoethanol) at 37°C for 45 min and adsorbance at 420 nm was measured. Activity due to background fusion typical of Cos-7 cells as well as background β-galactosidase expression was measured after cells were transfected with comparable amounts of vector alone and values obtained were subtracted from values obtained with cells expressing HN and wild-type F or mutant F proteins. Values obtained from cell extracts expressing HN and wild-type F protein were 5 to 6 times the background values.

Lipid mixing. The protocol used was similar to that previously described (Kemble *et al.,* 1993, 1994). Avian RBC (Crane Laboratories) were washed in PBS, resuspended in PBS, and incubated with 15 μg/ml R18 (Molecular Probes) for 30 min at room temperature in the dark. Three volumes of complete medium (DMEM with 10% fetal calf serum) was added and incubation was continued for 30 min. The RBC were washed 4 times in ice-cold PBS, resuspended in PBS containing $CaCl_2$ (0.01%), and added to transfected cells that had been washed in PBS with $CaCl_2$. Cells were incubated for 1 h at 37°C, washed in cold PBS containing $CaCl_2$, and visualized using a Nikon fluorescence microscope.

ACKNOWLEDGMENTS

This work was supported by Grants GM 37745 and AI 30572 from the National Institutes of Health.

REFERENCES

Baker, K. A., Dutch, R. E., Lamb, R. A., and Jardetzky, T. S. (1999). Structural basis for paramyxovirus-mediated membrane fusion. *Mol. Cell* **3,** 309–319.

Buckland, R., Malvoisin, E., Beauverger, P., and Wild, F. (1992). A leucine zipper structure present in the measles virus fusion protein is not required for its tetramerization but is essential for fusion. *J. Gen. Virol.* **73,** 1703–1707.

Carr, C. M., and Kim, P. S. (1993). A spring loaded mechanism for the conformational change of influenza hemagglutinin. *Cell* **73,** 823–832.

Chan, D. C., and Kim, P. S. (1998). HIV entry and its inhibition. *Cell* **93,** 681–684.

Chen, L., Gorman, J. J., McKimm-Breschkin, J., Lawrence, L. J., Tulloch, P. A., Smith, B. J., Colman, P. M., and Lawrence, M. C. (2001). The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. *Structure* **9,** 255–266.

Collins, P., and Mottet, G. (1991). Posttranslational processing and oligomerization of the fusion glycoproteins of human respiratory syncytial virus. *J. Gen. Virol.* **72,** 3095–3101.

Deng, R., Wang, Z., Mirza, A., and Iorio, R. (1995). Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. *Virology* **209,** 457–469.

Doms, R. W., Lamb, R. A., Rose, J. R., and Helenius, A. (1993). Folding and assembly of viral membrane proteins. *Virology* **193,** 545–562.

Furuta, R. A., Wild, C. T., Weng, Y., and Weiss, C. D. (1998). Capture of an early fusion-active conformation of HIV-1 gp41. *Nat. Struct. Biol.* **5,** 276–279.

Ghosh, J. K., Peisajavich, S. G., Ovadia, M., and Shai, Y. (1998). Structure–function study a heptad repeat positioned near the transmembrane domain of Sendai virus fusion protein which blocks virus–cell fusion. *J. Biol. Chem.* **273,** 27182–27190.

Hernandez, L. D., Hoffman, L. R., Wolfsberg, T. G., and White, J. M. (1996). Virus–cell and cell–cell fusion. *Annu. Rev. Cell Biol.* **12,** 627–661.

Hernandez, L. D., Peters, R. J., Delos, S. E., Young, J. A., Agard, D. A., and White, J. M. (1997). Activation of a retroviral membrane fusion protein: Soluble receptor-induced liposome binding of the ALSV envelope glycoprotein. *J. Cell Biol.* **139,** 1455–1464.

Hsu, M., Scheid, A., and Choppin, P. (1981). Activation of the Sendai virus fusion protein (F) involves a conformational change with exposure of a new hydrophobic region. *J. Biol. Chem.* **256,** 3557–3563.

Hu, X., Ray, R., and Compans, R. W. (1992). Functional interactions between the fusion protein and hemagglutinin-neuraminidase of human parainfluenza viruses. *J. Virol.* **66,** 1528–1534.

Joshi, S. B., Dutch, R. E., and Lamb, R. A. (1998). A core trimer of the paramyxovirus fusion protein: Parallels to influenza virus hemagglutinin and HIV-1 gp41. *Virology* **248,** 20–34.

Kemble, G. W., Danieli, T., and White, J. W. (1994). Lipid-anchored influenza hemagglutinin promotes hemifusion, not complete fusion. *Cell* **76,** 383–391.

Kemble, G. W., Henis, Y. I., and White, J. M. (1993). GPI- and transmembrane anchored influenza hemagglutinin differ in structure and receptor binding activity. *J. Cell Biol.* **122,** 1253–1265.

Lamb, R. A. (1993). Paramyxovirus fusion: A hypothesis of changes. *Virology* **197,** 1–11.

Lambert, D. M., Barney, S., Lambert, A. L., Guthrie, K., Medinas, R., Davis, D. E., Bucy, T., Erickson, J., Merutka, G., and Petteway, S. R. (1996). Peptides from conserved regions of paramyxovirus fusion (F) proteins are potent inhibitors of viral fusion. *Proc. Natl. Acad. Sci. USA* **93,** 2186–2191.

Matthews, T. J., Wild, C., Chen, C.-H., Bolognesi, D. P., and Greenberg, M. L. (1994). Structural rearrangements in the transmembrane glycoprotein after receptor binding. *Immunol. Rev.* **140,** 93–104.

McGinnes, L., Sergel, T., Reitter, J., and Morrison, T. (2001). Carbohydrate modifications of the NDV fusion protein heptad repeat domains influence maturation and fusion activity. *Virology* **283,** 332–342.

Rapaport, D., Ovadia, M., and Shai, Y. (1995). A synthetic peptide corresponding to a conserved heptad repeat domain is a potent inhibitor of Sendai virus–cell fusion: An emerging similarity with functional domains of other viruses. *EMBO J.* **14,** 5524–5531.

Reitter, J., Sergel, T., and Morrison, T. (1995). Mutational analysis of the leucine zipper motif in the Newcastle disease virus fusion protein. *J. Virol.* **69,** 5995–6004.

Sergel, T., McGinnes, L., and Morrison, T. (2000). A single amino acid change in the Newcastle disease virus fusion protein alters the requirement for HN protein in fusion. *J. Virol.* **74,** 5101–5107.

Sergel, T., McGinnes, L. W., and Morrison, T. G. (1993a). The fusion promotion activity of the NDV HN protein does not correlate with neuraminidase activity. *Virology* **196,** 831–834.

Sergel, T., McGinnes, L. W., Peeples, M. E., and Morrison, T. G. (1993b). The attachment function of the Newcastle disease virus hemagglutinin-neuraminidase protein can be separated from fusion promotion by mutation. *Virology* **193,** 717–726.

Sergel-Germano, T., McQuain, C., and Morrison, T. (1994). Mutations in the fusion peptide and heptad repeat regions of the Newcastle disease virus fusion protein block fusion. *J. Virol.* **68,** 7654–7658.

Skehel, J. J., and Wiley, D. C. (1999). Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell* **95,** 871–874.

Skehel, J. J., and Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: The influenza hemagglutinin. *Annu. Rev. Biochem.* **69,** 531–569.

Tan, K., Liu, J.-H., Wang, J.-H., Shen, S., and Lu, M. (1997). Atomic structure of a thermostable subdomain of the HIV-1 gp41. *Proc. Natl. Acad. Sci. USA* **94,** 12303–12308.

Tanabayashi, K., and Compans, R. W. (1996). Functional interaction of paramyxovirus glycoproteins: Identification of a domain in Sendai virus HN which promotes cell fusion. *J. Virol.* **70,** 6112–6118.

Tsurudome, M., Kawano, M., Yuasa, T., Tabata, N., Nishio, M., Komada, H., and Ito, Y. (1995). Identification of regions on the hemagglutinin-neuraminidase protein of human parainfluenza virus type 2 important for promoting cell fusion. *Virology* **213,** 190–203.

Wang, C., Raghu, G., Morrison, T., and Peeples, M. E. (1992). Intracellular processing of the paramyxovirus F protein: Critical role of the predicted amphipathic alpha helix adjacent to the fusion domain. *J. Virol.* **66,** 4161–4169.

Weissenhorn, W., Dessen, A., Calder, L. J., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1999). Structural basis for membrane fusion. *Mol. Membr. Biol.* **16,** 3–9.

Wiessenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997). Atomic structure of the ectodomain from HIV-1 gp-41. *Nature* **387,** 426–430.

Wild, T. F., and Buckland, R. (1997). Inhibition of measles virus infection and fusion with peptides corresponding to the leucine zipper region of the fusion protein. *J. Gen. Virol.* **78,** 107–111.

Yao, Q., and Compans, R. W. (1996). Peptides corresponding to the heptad repeat sequence of human parainfluenza virus fusion protein are potent inhibitors of virus infection. *Virology* **223,** 103–112.

Young, J. K., Hicks, R. P., Wright, G. E., and Morrison, T. G. (1997). Analysis of a peptide inhibitor of paramyxovirus (NDV) fusion using biological assays, NMR, and molecular modeling. *Virology* **238,** 291–304.

Young, J. K., Li, D., Abramowitz, M. C., and Morrison, T. G. (1999). Interaction of peptides with sequences from the Newcastle disease virus fusion protein heptad repeat regions. *J. Virol.* **73,** 5945–5956.

Zhao, X., Singh, M., Malashkevich, V. N., and Kim, P. S. (2000). Structural characterization of the human respiratory syncytial virus fusion protein core. *Proc. Natl. Acad. Sci. USA* **97,** 14172–14177.